THE DETERMINATION OF THE PHENOLIC COMPOSITION, ANTIOXIDATIVE ACTIVITY AND HEAVY METALS IN THE EXTRACTS OF *Calendula officinalis* L.

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The content of total phenols, flavonoids and antioxidant activities of different plant extracts of *Calendula Officinalis* L., used in Serbian traditional medicine were determined. The phenol content was determined by using UV-Vis measurements at 765 nm in the tested plant extracts obtained by standard extraction of *Calendula officinalis* L. The tested extracts, examined by DPPH method showed a high antioxidant activity that correlated significantly with the content of phenols and flavonoids. All tested extracts exhibited a strong scavenging activity against DPPH radicals (more than 96,8%). The contents of metals (Zn, Fe, Cu, Mn, Cd, Cr, and Pb) were determined by the flame atomic absorption spectrometer. Toxic metals, such as Cd, Cr, and Pb were not detected in the studied plant materials. The content of other metals in the plants and their extracts were low, except for iron. We examined the correlation of metals (Zn, Fe, Cu, Mn) and the phenolic compounds content in the extracts. The studied plants from the Southeast Serbia are suitable for the preparation of teas and herbal extracts due to the low content of toxic metals (Zn, Fe, Cu, and Mn), a high content of phenolic compounds and a high antioxidant activity.

Keywords: heavy metals, phenols, flavonoids, antioxidant activity, *Calendula officinalis* L.

Introduction

Polyphenol compounds are commonly found in both edible and inedible plants, and they have been reported to have multiple biological effects, including antioxidant activity [1]. Herbs are used on various occasions including medicine, nutrition, flavoring, in beverages, dyeing, as repellents, fragrances, in cosmetics [2]. Phenolic compounds, important constituents in many plants, have received considerable attention as potentially protective factors against cancer and heart diseases because of their antioxidant potency and their ubiquity in a wide range of commonly consumed foods of plants origin [3]. It is generally accepted that phenolic compounds behave as antioxidants as a result of the reactivity of the phenolic moiety. There are several mechanisms of the antioxidant activity, but it is believed that radical scavenging via hydrogen atom donation is the predominant mode of action. Other established antioxidant mechanisms involve radical complexing of pro-oxidant metals, as well as quenching through the electron donation and singlet oxygen quenching. Many studies have reported that phenolic compounds possess other biological activities such as anti-inflammatory, antiulcer, antispasmodic, antisecretory, antiviral, antidiarrhoeal, antitumor, etc. [4].

Humans consume and use different medicinal plants. Although medicinal plants are widely considered to be of lower risk compared to synthetic drugs, they are not

completely free from the possibility of toxicity or other side effects [5]. However, there is a considerable interest in identifying natural antioxidants as an alternative to synthetic medicines isolated from plants that protect them against free radical damage [6, 7]. Phenolic compounds from plants belong to the class of bioactive components with antioxidant activities. Plant phenols represent an important group of natural antioxidants (phenolic acids, flavones, isoflavones, flavan-3-ols, anthocyanins, proanthocyanidins, tannins, etc.), which show the antioxidant activity, antimutagenic, anticancer, anti-inflammatory, anti-ulcer and antimicrobial properties, and also decrease the risk of cardiovascular diseases [6, 7].

Although the effectiveness of medicinal plants is mainly associated with their constituents, it was found that their prolonged intake can cause health problems due to the possible presence of heavy metals [8]. During cultivation, plants can be easily contaminated with heavy metals. Also, the contamination of a herbal product is possible during processing. Because of the accumulation of heavy metals in plants, there is an increasing interest in the determination of their contents. For growth and good health, the human body requires both metals and nonmetals within certain permissible limits. Therefore, the determination of the content of elements in food and related products is essential for understanding

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their nutritive importance. Unfortunately, the presence of some metals in higher quantities in the body may have a toxic effect. [8-11]. Therefore, the control of heavy metals in medicinal plants and their products should be made in order to ensure the safety and efficacy of herbal products [12]. Plant, *Calendula officinalis* L. (flower) was selected because of the availability and existing studies on its medicinal use (anti-inflammatory, antidiabetic, antihypertensive, anticancer and antiviral activity) in the traditional Serbian medicine [13-16]. *Calendula officinalis* L. is a plant from the family Asteraceae. It has antibacterial and bactericidal effect and therefore it is used for treating wounds, psoriasis, etc. [17]. *Calendula officinalis* L. (flower) is known for its anti-inflammatory and anti-cancer properties [18].

It is selected for the present study because it is used as a traditional medicine in the Southeast Serbia for treating a cold, cough, catarrh, tonsillitis, and bronchitis.

There are no studies on the investigation of the extracts of this plant regarding the total content of phenolic compounds, their antioxidant activity and the concentrations of heavy metals.

Experimental

Preparation of materials

The plant material was collected in the phase of flowering from the natural habitats of the plant in the flowering stage, in the region of the Southeast Serbia in July 2013. The studied area is located in the surroundings of the city of Niš. Niš has about 300.000 inhabitants and represents one of the biggest cities in Serbia after Belgrade, but the industry in this area is very poorly developed. The sample sites were selected in accordance with the methods used in the European moss monitoring project [19]. A minimum distance of 300 m to major roads and larger settlements was required, a minimum distance of 100 m to minor roads and houses and a minimum distance of 5 m to the forest roads.

Determination of selected metals

The standard procedure for the determination of the selected metals described was followed for the preparation of the samples for the analysis [20]. The accurately weighed (2 g) sample was transferred into a silica crucible and kept in a muffle furnace for ashing at 450 °C for 3 h, and then 5 ml of 6 M HCl was added to the crucible. Care was taken to ensure that all the ash came into the contact with acid. Further, the crucible containing acid solution was kept on a hot plate and digested in order to obtain a clear solution. The final residue was dissolved in 0.1 M HNO₃ solution and made up to 50 ml. Working standard solutions were prepared by diluting the stock solution with 0.1 M nitric acid in order to check the linearity.

Preparation of herbal extracts

Dried plant material (flowers) of plant species was ground in a blender. The samples of 1 g of species were weighed from the ground and a homogenized herb and extracted with solvents listed in Table 1. The extraction was carried out for 15 min three times with 30, 20, and 20 ml of the solvent, respectively. The selected solvents were: ethanol, ethanol-water: 50-50, v/v (%), and water. The extraction was performed in an ultrasonic bath. The extracts were filtered through a Buchner funnel and filter paper (blue collar), transferred into a volumetric 100 ml flask and the same solvent was added to the mark. The contents of the selected metals in these plant extracts were determined by the AAS method.

Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, quercetin and AlCl₃ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent and sodium carbonate were purchased from Merck Chemical Suppliers (Darmstadt, Germany). Sodium chlorate buffer (pH 1.0) and acetate buffer (pH 4.5) were purchased from the same producer. All other chemicals used, HCl, HNO₃ (0.1 M HNO₃ standard solutions) including solvents of analytical grade were purchased from Sigma-Aldrich (GmbH, Steinheim, Germany). The working solutions were prepared from the basic solution (concentration 1000 mg/l for all metals), immediately before the analysis. For the preparation of standard solutions, high purity Milli-Q water was used which was purchased from Merck KGaA, Darmstadt, Germany. The glassware and polyethylene containers used for the analysis were washed with tap water, then soaked over the night in 6 M HNO₃ solution and rinsed several times with ultra pure water to eliminate the absorbance due to detergent.

Determination of the total phenolics

Total phenol contents in the extracts were determined by the modified Folin-Ciocalteu method [21]. An aliquot of the extracts (1 ml) was mixed with 0.5 ml of Folin-Ciocalteu reagent and 1.5 ml of the sodium carbonate solution (20%). Tubes were vortexed for 15 s and allowed to stay at 40 °C for 30 min in order to develop the color. Absorbance was then measured at 765 nm using the Hewlett Packard UV-VIS spectrophotometer. The total phenol content was expressed as mg g⁻¹ of gallic acid equivalent (GAE). The result of each assay was obtained from three parallel determinations.

Determination of total flavonoid contents

Total flavonoid contents were determined using the spectrophotometric method based on the formation of the flavonoid complex with aluminum [22]. The volume of 0.5 ml of 2% AlCl₃ methanol solution was added to 0.5 ml of the sample solution. After one hour of staying at room temperature, the absorbance was measured at 420 nm. The yellow color indicated that the extracts contained flavonoids. The total flavonoid content was calculated as

the concentration of quercetin ($\text{mg} \cdot \text{g}^{-1}$) using the equation based on the calibration curve.

Free radical scavenging activity

The free radical scavenging activity of the plant extracts was analyzed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [23, 24]. The antioxidant assay was based on the measurement of the loss of the color of the DPPH solution by the change of the absorbance at 517 nm caused by the reaction of DPPH with the tested sample. The reaction was monitored using a UV-VIS spectrophotometer. The plant extracts (0.2 ml) and 1.8 ml of freshly prepared DPPH in methanol (20 mg dm^{-3}) was put into a cuvette at room temperature. After 20 min of the incubation period at room temperature, the absorbance was read against a blank probe at 517 nm. The determinations were performed in triplicates. The inhibition of DPPH in percents (RSC%) of each plant extract sample was calculated from the decrease of the absorbance according to the relationship:

$$\text{RSC (\%)} = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100 (\%)$$

where A_{blank} is the absorbance of the control reaction, and A_{sample} is the absorbance of the tested sample.

Statistical analysis

The experimental results were expressed as the mean value $\pm$ standard error of the mean value of three replicates.[25].

Results and discussion

In our paper, the content of total phenols, flavonoids, heavy metals (Fe, Zn, Cu, and Mn) and antioxidant activities were determined in the investigated plant extracts of *Calendula officinalis* L. from the region of Southeast Serbia (Table 1). The content of total phenols in the extracts of the investigated plant was determined using the Folin-Ciocalteu method and was expressed as mg GAE g^{-1} of the fresh sample. (Table 1.)

Table 1. The comparative study of total phenols, flavonoids, the heavy metals content and antioxidant activities in the extracts of *Calendula officinalis* L.

Extract	Total phenols content ^a	Flavonoid content ^b	Antioxidant activity		Fe mg/kg	Zn mg/kg	Cu mg/kg	Mn mg/kg
			(RSC) in (%)	(RSC) ^c				
Plant					137.53 $\pm$ 2.75	18.15 $\pm$ 0.36	12.82 $\pm$ 0.26	24.38 $\pm$ 0.48
Water	45.13 $\pm$ 0.0	0.12 $\pm$ 0.00	85.22 $\pm$ 0.34	3.17 $\pm$ 0.17	16 $\pm$ 0.32	4.46 $\pm$ 0.09	1.50 $\pm$ 0.03	5.40 $\pm$ 0.11
Ethanol	31.86 $\pm$ 1.1	0.10 $\pm$ 0.00	27.31 $\pm$ 1.21	19.17 $\pm$ 0.04	76 $\pm$ 1.57	5.28 $\pm$ 0.10	2.40 $\pm$ 0.05	13.71 $\pm$ 0.27
Ethanol-water (50/50, v/v (%))	29.79 $\pm$ 0.7	0.17 $\pm$ 0.00	96.85 $\pm$ 0.26	0.038 $\pm$ 0.07	17 $\pm$ 0.34	4.42 $\pm$ 0.09	1.10 $\pm$ 0.02	3.60 $\pm$ 0.07

^aExpressed as mg of gallic acid g^{-1} of dry sample

^bExpressed as mg of quercetin g^{-1} of dry sample

^cExpressed as mg of quercetin g^{-1} of dry sample

In the case of the water extract, the total phenolic content of the investigated plant extracts ranged from 29.79 mg GAE g^{-1} of fresh plant petals for the ethanol-water: 50/50, v/v(%) to 45.13 mg GAE g^{-1} of the dry sample. The content of phenols was the highest in water extracts. The difference in the total content of phenolic compounds depended on the extraction solution.

The content of phenols represents a pharmacological characteristic of the plant. In the case of medicinal herbs, the concentration of phenols went from 0.23 to 2.85 mg GAE g^{-1} of the dry sample, whereas the concentration of phenols in the case of feeding plants went from 0.26 to 17.51 mg GAE g^{-1} of the dry sample. On the basis of the literature data, the highest content of phenolic compounds was found in the plants from genus *Origanum*, around 20 mg GAE g^{-1} of the dry sample. [26].

The content of total phenols in the flower of *Calendula officinalis* L. (UV spectrophotometric method, Folin-Ciocalteu method) was 15.12 mg/g of the sample and the content of total flavonoids (Markham) was 5.13 mg g^{-1} of the sample [27].

On the basis of numerous studies it is known that the

content of polyphenol compounds depends on the genotype, soil conditions and the difference in the plant ripening [28]. Also, the environmental conditions, like altitude, light, temperature, the content of feeding material in soil, can have an effect on the phenylpropanoid metabolism [29].

On the basis of the experimental results shown in Table 1, the content of total flavonoids in the extracts of the investigated plant was much lower than the phenol content. The content of total flavonoids is expressed in the form of mg of quercetin equivalent per gram of the dry sample (mg QE g^{-1}). The content of flavonoids was the highest in the ethanol and the lowest in the ethanol-water: 50/50, v/v(%) extract. These experimental results are expected because of the solubility of phenols, flavonoids and their glucosides in water.

The antioxidant activity of the extracts of the investigated plant was determined by using the DPPH method. The antioxidant activity of different extracts was investigated with the spectrophotometric method using the ability of the extract to catch stable (DPPH), (2,2-diphenyl-1-picrylhydrazyl) radicals. The antioxidant activity is

expressed in mg of quercetin-equivalents (QE), *i.e.* the solution of quercetin which shows the identical activity as the investigated extracts. The data are shown in Table 1. All the extracts exhibited a strong scavenging activity against DPPH radicals, from 27.31 to 96.35%. These experimental data are expected because of the solubility of flavonoids. DPPH radical scavenging abilities of the investigated extracts showed that the tested extracts contained molecules with the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Comparing our results for the total phenol content, flavonoids and the antioxidant activity of the investigated plant from the Southeast area of Serbia with the results of other authors, we can notice the firm agreement. The presence of heavy metals in the extracts can be explained by the possible complex formation that occurs between metal and organic compounds in the plant.

The presence of metals in plants is the result of the transfer of metals from the soil, water and atmospheric precipitation during growing. Their contents are generally lower due to the extraction by using different solvents.

Among the considered metal contents, the iron content was the highest (137.53 mg/kg plant). On the other side, the concentrations of Cu, Zn, and Mn were remarkably lower (between 12.82 and 24.38 mg/kg plants). The highest content of Fe was showed to be present in ethanol extracts of *Calendula officinalis* L. The quantity of other heavy metals, Cu, Zn, and Mn was present in the highest quantity in the ethanol solutions of the investigated plant. Ions of Cd, Pb, Cr, and Mn were not detected in the extracts. The presence of Fe, Cu, Zn, and Mn in the ethanol extracts can be explained by the possible complex formation occurring between metal and organic compounds in the plant. Minerals are inorganic substances present in all body tissues and fluids, and their presence is necessary for the maintenance of certain physicochemical processes which are essential to life [8, 9].

These include the calcification of bones, blood coagulation, neuromuscular activity, acid base equilibrium, enzyme activity, osmotic regulation.

The correlation of the content of total phenols, flavonoid compounds, heavy metal contents and antioxidant activity of the investigated extracts was analysed (Table 2.). It was possible to observe the correlations between metal ions and the level of flavonoids was high. The correlation of the levels of flavonoids and phenolic compounds with the antioxidant activity of the plant extracts was high, too.

The comparison of our results regarding the heavy metals content in the plant from the region of the Southeast Serbia with the results of other authors showed a considerable agreement. The content of copper in Palestinian plants varied from 7.06 to 19.19 mg/kg, and of zinc from 17.38 to 65.85 mg/kg [9]. The copper content in the black tea originating from the region of south India varied between 15.9 and 32.2 mg/kg [10]. In the plant from the region of the Southeast Serbia, the highest content was that of iron, while the contents of Zn, Cu, and Mn are significantly lower. Certain quantities of heavy metals in the plants were below the limit acceptable for the medicinal plant. Heavy metals, such as Cd, Cr, and Pb were not detected in the investigated plant. In the black tea samples originating from the region of Iran, the copper concentration was within the range from 17.59 to 32.80 mg/kg, and in the water extracts from 1.15 to 1.65 mg/kg [30]. The iron concentration in the medicinal plant from Turkey ranged from 2.45 to 107.4 mg/kg, zinc from 3.90 to 18.00 mg/kg, and copper from 2.45 to 8.10 mg/kg [31]. Comparing our results with the results of the authors from other countries it can be observed that the heavy metals content is similar or smaller. Based on these results we recommend the use of the water extracts with lower abundance of heavy metals.

Table 2. Correlation coefficients of total phenolic, flavonoids, the heavy metal content and antioxidant activity in the extracts of *Calendula officinalis* L..

-	Total phenols content ^a	Flavonoid content ^b	(RSC) ^c	Fe mg/kg	Zn mg/kg	Cu mg/kg	Mn mg/kg
Total phenols content ^a	1	0.1289	0.592	0.1614	0.1226	0.0088	0.0575
Flavonoid content ^b		1	0.6690	0.5047	0.5603	0.8021	0.6826
(RSC) ^c			1	0.9721	0.9875	0.9771	0.9998
Fe (mg/kg)				1	0.9969	0.9013	0.9672
Zn (mg/kg)					1	0.9320	0.9841
Cu (mg/kg)						1	0.9812
Mn (mg/kg)							1

^aExpressed as mg of gallic acid g⁻¹ of dry sample

^bExpressed as mg of quercetin g⁻¹ of dry sample

^cExpressed as mg of quercetin g⁻¹ of dry sample

Conclusion

The extracts obtained from the flowers of marigold (*Calendula officinalis* L.) contained a high quantity of polyphenolic compounds and exhibited good antioxidant activities. Phenolic compounds accounted mainly for the antioxidant activity of the plant extracts.

There are significant correlations of the antioxidant activity with the content of phenols and flavonoids. The contents of heavy metals (Fe, Zn, Cu, and Mn) were low, with the exception of Fe. Other heavy metals were not detected. The metal content in the extracts was lower than the content of metals in the plant.

These investigations are obligatory and they are recommended by the European standards in order to prevent poisoning by heavy metals.

The investigated plant from the Southeast Serbia region is suitable for the preparation of teas and herbal extracts due to the low content of heavy metals, the high content of phenolic compounds and a high antioxidant activity.

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Izvod

ODREĐIVANJE FENOLNOG SASTAVA, ANTIOKSIDATIVNE AKTIVNOSTI I TEŠKIH METALA U EKSTRAKTIMA NEVENA *Calendula officinalis* L.

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Sadržaj fenola, flavonoida i antoksidativne aktivnosti u različitim ekstraktima nevena, koji se koristi u narodnoj medicini u Srbiji su određeni. Sadržaj fenola, flavonoida i antioksidativna aktivnost određeni su primenom spektrofotometrijske metode, u ispitivanim ekstraktima, koji su pripremljeni standardnom metodom. Ispitivani ekstrakti su ispitani DPP metodom i pokazuju visoku antioksidativnu aktivnost koja je u značajnoj korelaciji sa sadržajem fenola i flavonoida. Svi ispitivani ekstrakti pokazuju veću antioksidativnu aktivnost od 96,8%. Sadržaj metala (Zn, Fe, Cu, Mn, Cd, Cr, and Pb) određen je primenom atomske absorpcione spektrometrije. Toksični metali, kao što su Pb, Cr i Cd, nisu detektovani u ispitivanim ekstraktima. Sadržaj ostalih metala je nizak sa izuzetkom gvožđa. Određena je korelacija sadržaja metala i sadržaja fenolnih jedinjenja u ispitivanim ekstraktima. Ispitivani ekstrakti nevena (*Calendula officinalis* L.) su pogodni za upotrebu jer imaju nizak sadržaj metala, visok sadržaj fenolnih jedinjenja i visoku antioksidativnu aktivnost.

Ključne reči: teški metali, fenoli, flavonoidi, antioksidativna aktivnost, *Calendula officinalis* L.